FLUORIDE ION REGENERATION OF CYCLOSARIN (GF) FROM MINIPIG TISSUE AND FLUIDS FOLLOWING WHOLE BODY GF VAPOR EXPOSURE

Edward M. Jakubowski; Jeffery M. McGuire; Ronald A. Evans; Stanley W. Hulet; Robert J. Mioduszewski; Sandra A. Thomson

US Army ECBC, Edgewood, MD 21010-5424

ABSTRACT

Recent developments to improve nerve agent biomarker techniques include methods for measuring fluoride regenerated Sarin (GB) in blood and tissue. Our efforts extend the fluoride ion regeneration method to be able to determine cyclosarin (GF) in red blood cells, plasma, and tissue of minipig blood samples after whole body exposure to GF at miosis levels. Blood samples were taken serially before, during, and after whole body GF exposure from the minipig via venous catheter allowing agent exposure profiles to be generated. After processing the samples with fluoride ion and extracting with C-18 solid phase extraction cartridges the ethyl acetate extract was analyzed by GC/MS. The GC/MS method utilized an autoinjector, a large volume injector port (LVI), positive ion ammonia chemical ionization detection in the SIM mode, and a $^{2}H_{11}$-GF stable isotope internal standard. Results indicated that the method range was 10-1000 pg on column. The detection limit was 3 pg of GF on column despite the complexity of the red blood cell/tissue matrix. Conditions that needed to be optimized for the LVI included injection volume, initial temperature, pressure, and flow rate. The regenerated GF (R-GF) profiles differ greatly from the regenerated GB (R-GB) profiles in the minipig at similar exposure levels. The onset of the appearance of R-GF in the blood seems to be delayed and maximum levels are reached at much later times as compared to GB exposures. The rate of R-GB production was 5-10 times greater than that of R-GF at equimolar exposures.

1. INTRODUCTION

The acute toxicity of nerve agents and for the related organophosphorus pesticides is caused by irreversible inhibition of acetylcholinesterase, which produces an excess of the neurotransmitter acetylcholine in central, peripheral, and neuromuscular synapses. Typically, organophosphorus inhibitors of acetylcholinesterase and similar esterases react with the serine residue at the active site to produce the O-alkyl methylphosphonylated enzyme and a free leaving group. For cyclosarin and sarin, the leaving group is a fluoride ion. Over time, the O-alkyl methylphosphonylated enzyme undergoes a process known as “aging” in which the O-alkyl group is removed by hydrolysis to produce the highly stable methylphosphonylated enzyme. Overall, the toxic results of excess acetylcholine include miosis, sweating, excessive salivation, muscle fasciculation, seizure, respiratory failure, and death.

Exposure assessment and risk assessment are critical with respect to preventing contamination, evaluating extent of exposure, determining the care of exposure individuals, and preventing secondary exposures. Historically, exposure assessment was limited to criteria based on medical observations of the signs and symptoms of cholinergic crisis and blood cholinesterase inhibition. Risk assessment is based on the exposure data collected from animal experiments and human records when available. Cholinesterase inhibition has been a useful exposure assessment tool because it was readily available and revealed all inhibitors including relevant commercial pesticides. However, it was impossible to say that cholinesterase inhibition was due to a specific agent. Furthermore, variations in cholinesterase activity within individuals and populations would make minor exposures difficult to detect (1).

In addition to reacting with cholinesterases, nerve agents such as cyclosarin and sarin hydrolyze under physiological conditions to produce alkyl methylphosphonates. Exposure assessment and verification methods using either gas chromatography (GC) or liquid chromatography (LC) were developed for sarin, cyclohexylsarin, VX, and related compounds that could analyze for their alkyl methylphosphonate metabolites in blood, tissue, and/or urine (2, 3, 4). For example, the hydrolysis product of sarin, isopropyl methylphosphonic acid (IMPA), was found in the serum, brain tissue, and urine of the Tokyo subway victims (5,6,7,8). Methods based on detection of alkyl methylphosphonates have been useful because they indicated the individual was

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 NOV 2006	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Fluoride Ion Regeneration Of Cyclosarin (Gf) From Minipig Tissue And Fluids Following Whole Body Gf Vapor Exposure	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **US Army ECBC, Edgewood, MD 21010-5424**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM002075., The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**8**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

exposed to a chemical that yields an alkyl methylphosphonate. However, IMPA analysis is non-specific and IMPA will also be produced by O, O'-diisopropyl methylphosphonate (11) and bis (O-isopropyl methyl) pyrophosphate (12). Analysis of IMPA by GC required derivatization, which is time consuming and adds an extra layer of complexity to the sample preparation. Alkyl methylphosphonates metabolites are very water soluble and are eliminated rapidly by the kidneys (2, 3). Therefore, exposure assessments based on these metabolites are affected by parameters such as clearance rate, which adds an extra measure of variability to the overall assessment. Evidence of sarin exposure in the Tokyo subway victims was also provided by the electrospray tandem mass spectrometric analysis of phosphonylated nonapeptides from serum butyrylcholinesterase after pepsin digestion (9). This method has the ability to detect exposure from nerve agent inhibited butyrylcholinesterase after aging has occurred. Aging rate varies from agent to agent and from species to species. The aging process may take anywhere from minutes to hours depending on the agent and species. Another recent analytical development to improve the specificity and sensitivity of exposure assessment included a GC-MS method for measuring fluoride ion regenerated alkyl methylphosphonofluoridates from human and animal plasma butyrylcholinesterase. The regeneration process involves nucleophilic attack of fluoride ion on the agent phosphonylated enzyme before aging has occurred to release an alkyl methylphosphonofluoridate (nerve agent). The free nerve agent is quickly extracted from the matrix and analyzed by gas chromatography-mass spectrometry (GC-MS). This method was able to verify sarin exposure in the plasma samples of certain victims of the Aum Shinrikyo cult terrorist attacks in a Matsumoto neighborhood in 1994 and the following year in the Tokyo subway (10). Of the methods available to assess nerve agent exposure, the fluoride ion regeneration method has demonstrated some unique advantages over cholinesterase inhibition assays and the alkyl methylphosphonates methods (13). The fluoride ion regeneration method has the advantage of greater specificity because in order to yield positive results the agent must have first reacted with the enzyme, which indicated the original agent had a suitable leaving group to react with the cholinesterase. Also, this method has been recently applied to blood samples from sarin exposed guinea pigs as a dosemetric to calculate equivalent doses for inhalation and systemic routes of agent exposure (17). However, it would still be impossible to be completely certain of the identity of the original agent without knowing the leaving group. In regards to sample preparation, the fluoride ion method has generated volatile nerve agents which negated the need for derivatization before GC analysis.

This paper has presented an extension of the fluoride ion regeneration method for determining cyclosarin in red blood cell samples and other tissues, which has incorporated solid-phase extraction (SPE), ammonia CI in the positive ion mode, a large volume injector (LVI) with an autosampler, and a stable isotope internal standard. Performance characteristics established included: optimizing LVI parameters, identifying the useful reportable range, estimating the accuracy and precision, and determining detection limits. Development and validation was performed using Gottingen minipig blood and tissue samples. The Gottingen minipigs are being explored as a model of nerve agent inhalation exposure. They share many important anatomical and physiological characteristics with humans (14). One of these similarities was the near absence of circulating carboxylesterase in the blood. Carboxylesterase, which is present in rat blood, has been shown to react with nerve agents and can affect the ability to correlate interspecies sensitivities to nerve agents (15). In addition, the results from the whole-body GF minipig exposures are compared to GB minipig exposures at similar concentrations and exposure durations.

2. METHODS

Whole-body GB and GF exposures of minipigs of 10, 60, and 180 minutes were conducted in a 1000 L chamber using concentrations ranging from 0.01 to 30 mg/m^3. No anesthetic, pretreatment or post treatment drugs were given to test animals in order to simulate realistic exposure conditions. Blood samples were taken serially before, during, and after exposures via venous catheter allowing agent exposure profiles to be generated. All blood samples were collected in standard EDTA (ethylenediaminetetraacetic acid) containing tubes and then centrifuged at 15000 rpm for 5 minutes to separate the plasma from the red blood cells. Plasma was frozen until analysis and RBCs were refrigerated at 5^{o}C. RBC and plasma samples were extracted using C_{18} SPE columns (200 mg Sep-Pak, Waters Associates, Millipore

Corp., Milford, MA) which were first conditioned with 1 mL each of ethyl acetate, followed by isopropanol, and then pH 3.5 acetate buffer. For RBC samples, 1 mL of acetate buffer, 200 µL of KF solution (6M), and $^2H_{11}$-GF stable isotope internal standard were added and the mixture was vortexed for 10-20 seconds, followed by centrifugation at 15000 rpm for 5 minutes. The sediment at the bottom of the microvial was re-suspended with 750 µL of acetate buffer and 200 µL of KF solution. This mixture was vortexed followed by centrifugation at 15000 rpm for 5 minute and the resulting liquid was then added to the original solution. Fifteen minutes after the original addition of buffer and KF, the combined reaction mixture was allowed to drain through the conditioned SPE column under a gentle vacuum. After complete draining, the SPE column was washed with 500 µL of acetate buffer and allowed to dry using a light vacuum to pull air through the column for 3 minutes. The analytes were eluted with 1 mL of ethyl acetate that was collected and dried over anhydrous sodium sulfate. The ethyl acetate was removed from the collection tube and filtered through a 0.2 µm nylon Acrodisc syringe filter (Pall Gelman Laboratory, Ann Arbor, MI) into a GC autosampler vial for analysis. Injections of 50 µL of extract were made by autoinjector into the LVI (Agilent Technologies, model PTV, Wilmington, DE) using the following parameters: initial temp 0oC, initial time 5.1 min, final temp 260oC, rate 720oC/min, vent time 5.00 min, vent flow 300 mL/min, purge flow 50 mL/min, purge time 7.85 min. A Rtx-1701 (Restek Inc., Bellefonte, PA) column was used with a flow rate of 3 mL/min (63 cm/sec). Mass spectrometric detection (Agilent Technologies model 5793 MSD, Wilmington, DE) was by chemical ionization with ammonia reagent gas in the positive ion mode using the m/z 198/209 ammonia adduct ion ratio (GF/$^2H_{11}$-GF) for quantification and the m/z 215 (GF) and 226($^2H_{11}$-GF) ions as qualifiers. Linear internal standard calibration curves for GF were generated from 10-1000 pg using standards in ethyl acetate.

3. RESULTS AND DISCUSSION

Results indicated that the method range was 10-1000 pg on column. The detection limit was 3 pg of GF on column despite the complexity of the red blood cell/tissue matrix. Conditions that needed to be optimized for the LVI included injection volume, initial temperature, pressure, and flow rate. The regenerated GF (RGF) profiles differ greatly from the regenerated GB (RGB) profiles in the minipig especially after ten minute exposures at similar chamber concentrations as demonstrated in Figure 1. The onset of the appearance of RGF in the blood was delayed and maximum levels are reached at much later times as compared to GB exposures. In addition, the ten minute GF exposures produced a biphasic curve for RGF in RBC samples. Quantities of RGF were recovered from minipig blood during the first several hours of inhalation exposure that were one-fifth to one-tenth of RGB recovered at equimolar exposure concentrations and equal exposure duration times. Figure 2 compares the GB and GF rates of absorption versus exposure concentration. Each point represents the linear slope of RGB or initial RGF concentrations over time generated during a swine exposure versus the concentration used for the exposure. Assuming the amount of regeneration product found in the blood represents the absorbed dose at any particular time during exposure, GB is absorbed almost ten times faster than GF in swine and the absorption rate of both nerve agents is proportional to exposure concentration.

Inhibition of blood cholinesterase activity is typically used to monitor nerve agent exposure even though the correlation between enzyme activity and effect or target tissue activity is not always evident (16). Figure 3 compares the RBC acetylcholinesterase activity to the RGF concentration in the blood during a 10 minute minipig whole-body GF exposure (6.65 mg/m^3). Only the RGF concentration can be used to follow the exposure past 6 minutes because the AChE activity has dropped to near zero by that time although GF continues to be absorbed into the blood.

The performance of the RGB and RGF methods with regards to tissue samples is highlighted in Figures 4 and 5. Figure 4 compares the RGB and RGF recovery at miosis levels of exposure for RBC, lung, liver, kidney, and eye. Figure 5 compares the agents at lethal levels in the same tissues. At miosis levels both GB and GF yield similar amounts of agent in the tissues. However, at lethal levels approximately three times more GB is recovered in lung tissue compared to GF even though the GF exposure concentration was around 1.5 times greater. Given the lower volatility and water solubility of GF it is potentially scrubbed by the upper airway to a greater degree than GB resulting in lower levels in the lungs. More work is needed to

define and explain the differences in distribution between these two agents.

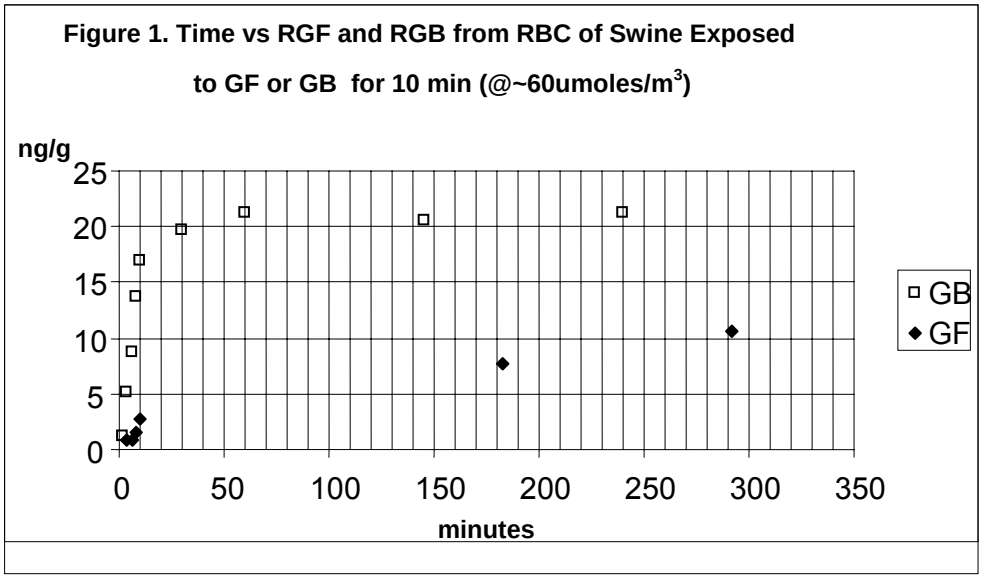
4. SUMMARY AND CONCLUSIONS

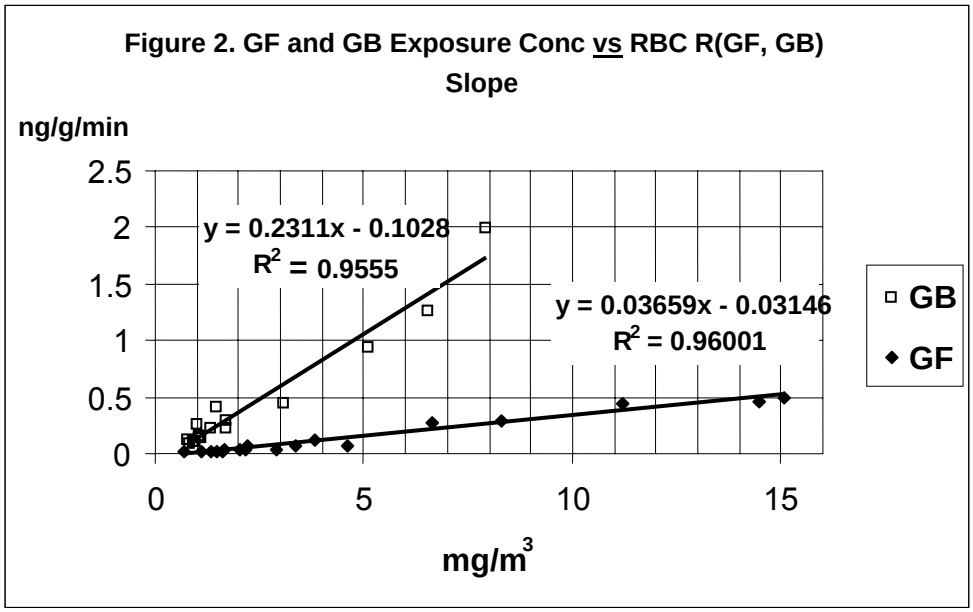
The isotope dilution LVI method is sensitive enough to quantify RGB and RGF in blood and tissue after lethal or miosis level exposure. The rates of RGB and RGF production are proportional to exposure levels. However, the respiratory uptake of GF as reflected by RBC RGF levels appears to be much slower than GB. Maximum levels of RGF were seen approximately 5 hours after the beginning of the exposure for all levels of exposure to date while R-GB maximum is reached soon after exposure stops. Similar intravenous doses produce twice as much R-GB as R-GF on a molar basis. At miosis levels, GB and GF produce similar amounts of R-agent in the tissues analyzed. However, R-GB is found in greater quantities in lung tissue at lethal levels.

5. REFERENCES

1. K.B. Augustinsson. The normal variation of human blood cholinesterase activity. *Acta Physiol Scand*. **35**: 40-52 (1955).
2. M.L. Shih, J.R. Smith, J.W. McMonagle, T.W. Dolzine, and V.C. Gresham. Detection of Metabolites of toxic alkymethylphosphonates in biological samples. *Biol. Mass Spectrom*. **20**:717-723 (1991).
3. M.L. Shih, J.W. McMonagle, T.W. Dolzine, and V.C. Gresham. Metabolite pharmacokinetics of soman, sarin and GF in rats and biological monitoring of exposure to toxic organophosphorus agents. *J. Appl. Toxicol*. **14**: 195-199 (1994).
4. W.J. Driskell, M. Shih, L.L. Needham, and D.B. Barr. Quantitation of organophosphorus nerve agent metabolites in human urine using isotope dilution gas chromatography-tandem mass spectrometry. *J. Anal. Toxicol.* **26**:6-10 (2002).
5. D. Noort, A.G. Hulst, D.H.J.M. Platenburg, M Polhuijs, H.P. Benschop. Quantitative analysis od O-isopropyl methylphosphonic acid in serum samples of Japanese citizens allegedly exposed to sarin: estimation of internal dosage. *Arch. Toxicol* **72**: 671-675 (1998).
6. Y. Matsuda, M. Nagao, T. Takatori, H. Niijima, M. Nakajima, H Iwase, M. Kobayashi, and K. Iwadate. Detection of the sarin hydrolysis product in formalin-fixed brain tissues of victims of the tokyo subway terrorist attack. *Toxicol. Appl. Pharmacol*. **150**:310-320 (1998).
7. M. Minami, D-M. Hui, M. Katsumata, H Inagaki, and C.A. Boulet. Method for the analysis of the methylphosphonic acid metabolites of sarin and its ethanol-substituted analog in urine as applied to the victims of the Tokyo sarin disaster. *J. Chromatog. B* **695**: 237-244 (1997).
8. T. Nakajima, K. Sasaki, H. Ozawa, Y. Sekijima, H. Mortia, Y. Fukushima, and N. Yanagisawa. Urinary metabolities of sarin in a patient of the Matsumoto sarin incident. *Arch.Toxicol*. **72**: 601-603 (1998).
9. A. Fidder, A.G. Hulst, D. Noort, R. de Ruitter, M.J. van der Schans, H.P. Benschop, and J.P. Langenberg. Retrospective detection of exposure to organophosphorus anti-cholinesterases: mass spectrometric analysis of phosphylated human butyrylcholinesterase. *Chem. Res. Toxicol*. **15**: 582-590 (2002).
10. M. Polhuijs, J.P. Langenberg, and H.P. Benschop. New method for retrospective detection of exposure to organophosphorus anticholinesterases: Application to alleged sarin victims of Japanese terrorists. *Toxicol. Appl. Pharmacol*. **146** :156-161 (1997).
11. G.W Ivie. Fate of diisopropyl methylphosphonate (DIMP) in a lactating cow. *Bull. Environ. Contam. Toxicol*. **24**: 40-48 (1980).
12. M. Nagao, T. Takatori, Y. Matsuda, M. Nakajima, H. Niijima, H. Iwase, K. Iwadate, and T. Amano. Detection of sarin hydrolysis products from sarin-like organophosphorus agent-exposed human erthrocytes. *J. Chromatog. B* **701**: 9-17 (1997).
13. EM Jakubowski, JM McGuire, RA Evans, JL Edwards, SW Hulet, BJ Benton, JS Forster, DC Burnett, WT Muse, K Matson, CL Crouse, RJ Mioduszewski, SA Thomson. Quantification of Fluoride Ion Released Sarin in Red Blood Cell Samples by Gas Chromatography-Chemical Ionization Mass Spectrometry Using Isotope Dilution and Large Volume Injection. J. Analytical Toxicology Vol. **28**(5), 357-363 (2004).
14. R.F. Witkamp and M Monshouwer. Pharmacokinetics in vivo and in vitro in swine. *Scand. J. Lab. Anim. Sci. Suppl. 1* **25**: 45-56 (1998).

15. D.M. Maxwell, K.M. Brecht, and B.L. O'Neill. The effect of carboxylesterase inhibition on interspecies differences in soman toxicity. *Toxicol. Letters* **39**: 35-42 (1987).
16. B. Dementi. Cholinesterase Literature Review and Comment. *Pesticides, People and Nature*, **1**(2):59-126 (1999).
17. S. Thomson, R. Mioduszewski, S. Reutter, D. Sommerville, S. Hulet, B. Benton, M. Jakubowski, C. Whalley, T. Shih, L. Lumley, S. DeFord, R. Kan, M. Roberson, M. Sipos, R. Genovese, J. Petras, J. Gearhart, K. Yu, P. Robinson, S. Channel. Low Level Chemical Warfare Agent Toxicology Research Program -FY04 Report and Analysis. Technical Report No.: AFRL-HE-WP-TR-2005-0054.





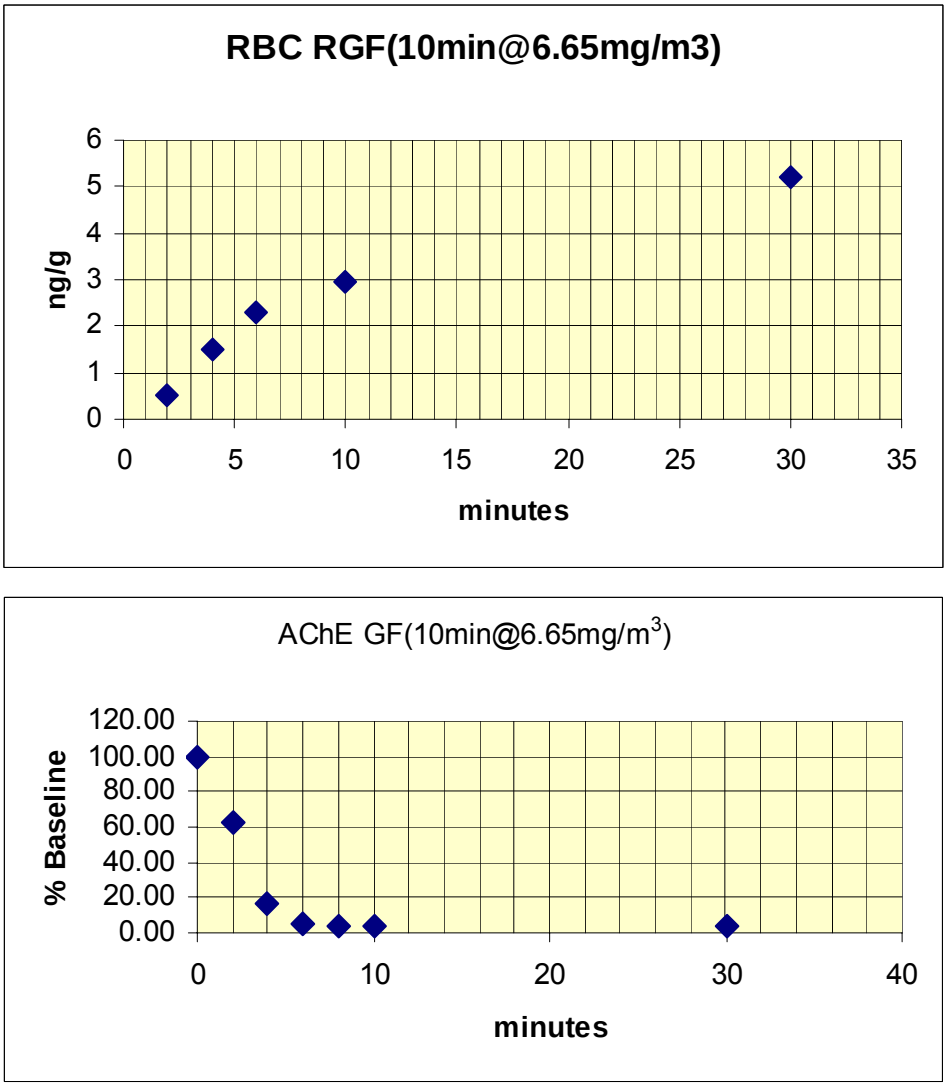


Figure 3: RGF vs AChE Activity

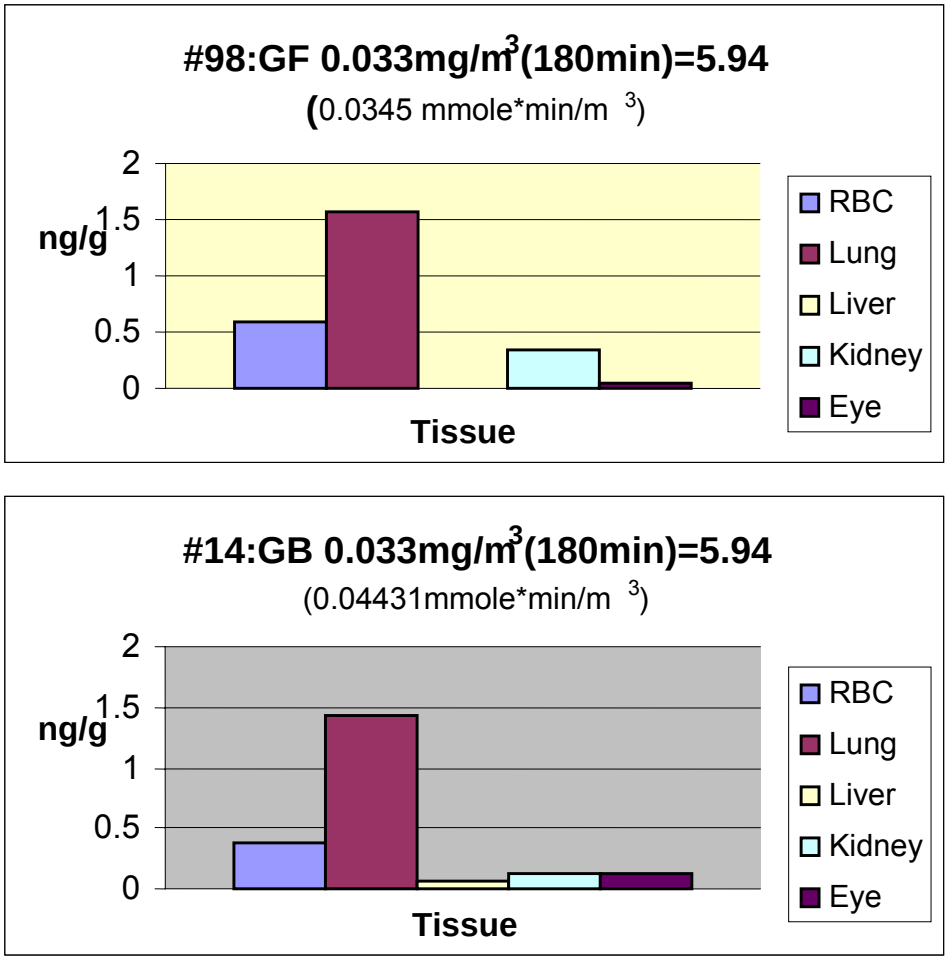


Figure 4. Miosis Level Exposure Tissue Comparison

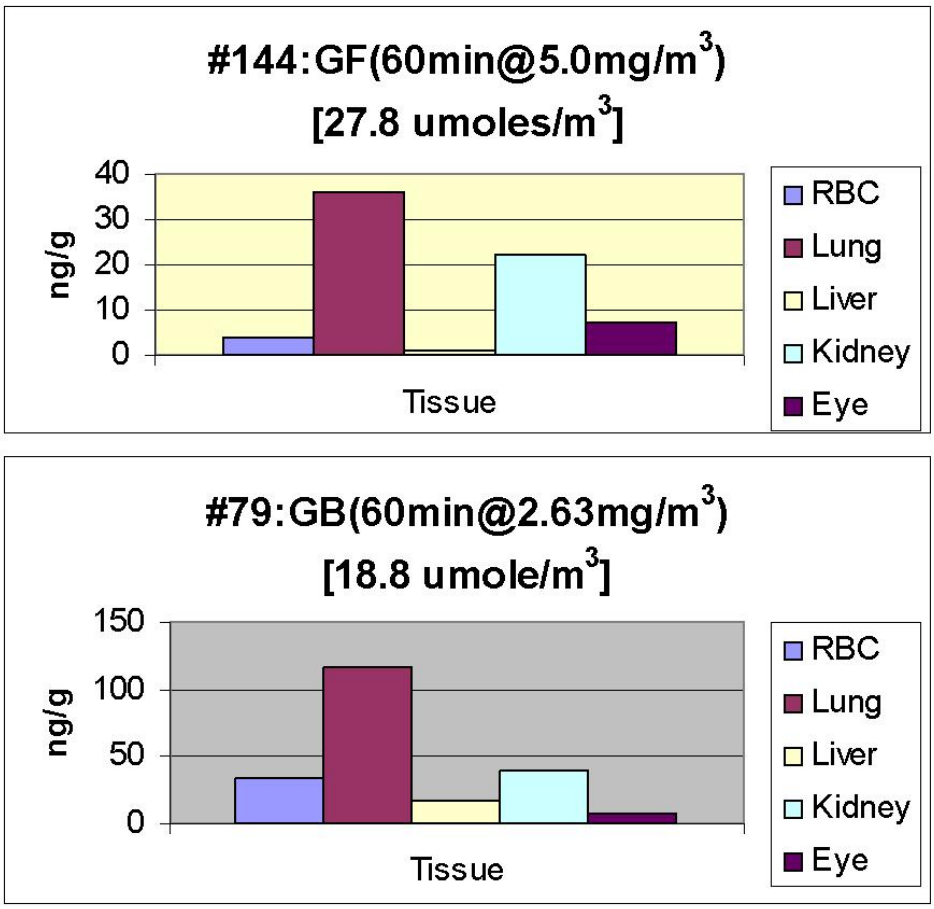


Figure 5. Lethal Level Exposure Tissue Comparison.